Mixed infection with Beijing and non-Beijing strains in pulmonary tuberculosis in Taiwan: prevalence, risk factors, and dominant strain

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Abstract

Patients with pulmonary tuberculosis (TB) can be simultaneously infected with different strains of *Mycobacterium tuberculosis* (mixed infection). We investigated the prevalence and risk factors of mixed infection by Beijing and non-Beijing strains in pulmonary TB patients in Taiwan. We developed a quantitative PCR method to simultaneously detect the presence of Beijing and non-Beijing strains. A total of 868 pretreatment samples (from 868 patients), including 563 sputum samples smear-positive for acid-fast bacilli and 305 liquid medium samples culture-positive for mycobacteria, were tested. Medical records of patients with culture-confirmed pulmonary TB were reviewed. The detection limit of our quantitative PCR method was five copies of target sequences. With mycobacterial culture result as the reference standard, the sensitivity and specificity of our quantitative PCR method were 95% and 98%, respectively. *M. tuberculosis* strains were isolated in 466 samples, of which 231 (49.6%) were infected with a Beijing strain. Another 14 patients (3.0%) had mixed infection, with the Beijing strain being the dominant strain in 13 (93%). Age <25 years with pulmonary cavities was associated with mixed infection. In patients infected with non-Beijing strains, the bacterial load of non-Beijing strains was lower among those with mixed infection than among those without. Our quantitative PCR method was accurate in detecting Beijing and non-Beijing strains in smear-positive sputum and culture-positive liquid medium samples. Mixed infection was present in pulmonary TB patients (3.0%), especially in those aged <25 years with pulmonary cavities. Beijing strains seem to be more dominant than non-Beijing strains in patients with mixed infection.

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Introduction

It has been commonly thought that tuberculosis (TB) is caused by a single strain of *Mycobacterium tuberculosis* [1,2]. However, studies have found that patients may be simultaneously infected with different strains (mixed infection) [3,4]. By amplifying and detecting DNA sequences of Beijing and non-Beijing strains, a study in Cape Town showed that 19% of all patients with TB were simultaneously infected with one Beijing and one non-Beijing strain, and that this occurred more frequently in retreatment cases (23%) [5]. Several other genotypic approaches based on detecting genomic regions specifically deleted in the Beijing strains have also been utilized to identify Beijing strain and mixed infection in clinical samples [6–8]. The presence of mixed infection can lead to conflicting results of drug susceptibility testing if both drug-susceptible and drug-resistant *M. tuberculosis* strains are present [9–11]. Drug-resistant strains may not be detected initially if susceptible strains are predominant, but may outnumber susceptible strains at a later point in time during anti-TB treatment. Furthermore, it was observed that a drug-susceptible strain re-emerged in patients with multidrug-resistant (MDR) TB treated with second-line anti-TB drugs [12]. Therefore, undetected drug-resistant strains in

the presence of drug-susceptible strains may result in unfavourable treatment outcomes.

The prevalence and risk factors of mixed infection in tuberculous populations have rarely been investigated, mainly because of technical difficulties. In Taipei, Taiwan, Beijing strains account for 50% of the clinical isolates of *M. tuberculosis* [13]. Therefore, the magnitude of mixed infection could be investigated by simultaneous detection of one Beijing and one non-Beijing strain. As the incidence of TB in Taipei is not as high as that in the study sites of Cape Town, we thought that mixed infection in TB is less frequent in Taipei, but hypothesized that it was not undetectable. Therefore, we conducted a study to investigate the prevalence and risk factors of simultaneous infection with Beijing and non-Beijing strains in patients with pulmonary TB by using a real-time quantitative PCR (Q-PCR) method. We report the results of this study.

Materials and Methods

Materials and protocols

This prospective study was conducted in a 700-bed medical centre in northern Taiwan. Pretreatment sputum samples from patients suspected of having TB that were either smear-positive for acid-fast bacilli or culture-positive for mycobacteria were collected from July 2007 to December 2008 in the mycobacteriology laboratory, which is a regional reference laboratory whose quality is periodically assessed by the national reference laboratory of the Taiwan Centers for Disease Control. Only one specimen was collected from each patient.

Mycobacteriological studies were performed as previously described (see Data S1) [14,15]. For sputum samples that were smear-positive for acid-fast bacilli, or culture-positive in liquid medium, we immediately extracted genomic DNA [16] and performed a PCR test with the Cobas Amplicor MTB system (Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer's instructions. The results of the PCR test were analysed with the Cobas Amplicor Analyzer (Roche Instrument Center AG, Rotkreuz, Switzerland).

Medical records of patients with pulmonary TB confirmed by both mycobacterial culture and Q-PCR test were reviewed to obtain the demographic data, history of previous TB, and chest radiographic findings.

Procedures of real-time Q-PCR

For each extracted genomic DNA, a Q-PCR test was also performed to detect mixed infection, defined as the simultaneous presence of a Beijing and a non-Beijing strain. If the Q-PCR test revealed mixed infection, we then performed a PCR with the same primer sets, and sequenced the amplicons on an ABI Prism 3730 DNA sequencer with a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) to confirm their presence.

Two primer sets and probes were designed to detect the Beijing and non-Beijing strains of *M. tuberculosis* in the samples (see Fig. S1). The primer sequences for the Beijing strains were complementary to the 3′-end of the IS*6110* element and Rv2820. A positive Q-PCR signal indicated the presence of an IS*6110* insertion in Rv2820, which is unique to the Beijing evolutionary lineage [17]. The primer sequences for non-Beijing strains were complementary to Rv2819. The standard curve for calculating the number of copies of each DNA sequence in the sample was generated with different concentrations of cloned plasmid containing the target sequence (see Data S1).

To assess the reproducibility of Q-PCR, two independent experiments were performed for each sample.

Minimizing laboratory cross-contamination

Several steps were applied to minimize the possibility of laboratory cross-contamination. First, fewer than five samples were processed at the same time. Second, acid-fast smears, mycobacterial cultures, DNA extraction and Cobas Amplicor MTB assays were performed in a biosafety level 2-plus laboratory, and the Q-PCR was performed in a biosafety level 2 laboratory. For each Q-PCR reaction, DNA from two different Beijing and two different non-Beijing strains, confirmed by spoligotyping [18], were used as positive controls, and de-ionized water as negative control.

Statistical analysis

The reproducibility of the Q-PCR was evaluated by applying Pearson's correlation and *t*-test. Correlation between the results of Cobas Amplicor and Q-PCR was evaluated by calculating the kappa coefficient. Because of the potentially non-linear effect of age, the spline smoothing model was applied to evaluate its impact on mixed infection. Multivariate logistic regression analysis was used to identify risk factors of mixed infection, and linear regression analysis to identify factors influencing mycobacterial load (see Data S1). A two-sided p-value <0.05 was considered to indicate significance. All analyses were performed with SAS software (Version 9.1.3; SAS Institute, Cary, NC, USA).

Results

A total of 868 samples, including 563 smear-positive sputum samples and 305 culture-positive liquid medium samples, col-

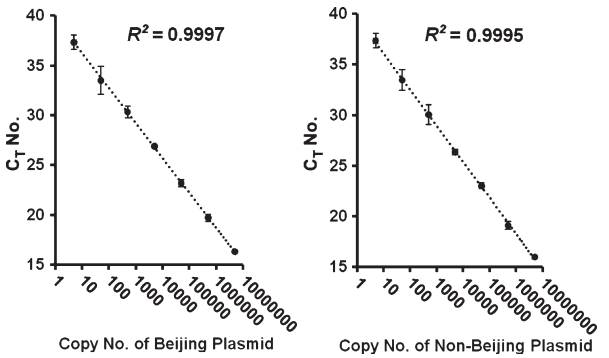

FIG. 1. The good correlation between the cycle threshold (C_T) number in the quantitative PCR and the amount of the Beijing plasmid (left) or non-Beijing plasmid (right). Each data point and error bar represent the average and 95% CI of the C_T numbers when corresponding copies of one plasmid were mixed with seven different copies (5, 50, 500, 5000, 50 000, 500 000, or 5 000 000) of the other plasmid.

lected from 868 patients were tested for the presence of mixed infection. Of the 868 samples, 486 (56.0%) were culture-positive for *M. tuberculosis* and 382 were either culture-negative (N = 261) or non-TB mycobacteria (N = 121).

Stability and reproducibility of the Q-PCR method

Fig. 1 shows that the correlation between the cycle threshold number in the Q-PCR and the amount of either the Beijing or non-Beijing DNA construct was good, despite the concomitant presence of the other DNA construct in a different amount (R^2 = 0.9997 and R_2 = 0.9995, respectively).

The Pearson correlation between the results of the two independent Q-PCR experiments for every sample was 0.999 for both Beijing and non-Beijing strains. The copy numbers calculated in two independent experiments for each sample were not significantly different for both strains (the p-values of independent-sample *t*-tests were 0.906 and 0.921, respectively).

Correlation between Q-PCR and Cobas Amplicor for the detection of *M. tuberculosis*

Table 1 shows the comparison of Q-PCR with Cobas Amplicor for the detection of *M. tuberculosis* in the 868 samples. With culture result as the reference standard, the Q-PCR yielded a false-negative result in 19 sputum samples and five liquid medium samples. Four sputum samples and one liquid medium sample had a false-positive Q-PCR result for Beijing strains (1196, 678, 262, 31 and 61 copies, respectively). Another sputum sample had a false-positive Q-PCR result for non-Beijing strains (838 copies).

The results of Q-PCR correlated well with those of Cobas Amplicor (the kappa coefficient was 0.896 for sputum samples, 0.946 for liquid medium samples, and 0.914 for both). With culture result as the reference standard, the Q-PCR method had an overall sensitivity and specificity higher than 95% (Table 1).

Prevalence and risk factors for mixed infection

Among the 486 samples that were culture-positive for *M. tuberculosis*, 466 were positive with the Q-PCR test. Of the 466 specimens, 292 (62.7%) were smear-positive sputum samples and 174 (35.8%) were culture-positive liquid medium samples (Table 2); 40 (8.5%) contained MDR *M. tuberculosis* strains, 45 (9.7%) contained resistant but not MDR *M. tuberculosis* strains, and 381 (81.8%) contained susceptible strains; 231 (49.6%) contained Beijing strains, 221 (47.4%) contained non-Beijing strains, and 14 (3.0%) showed mixed infection. The mean age of the 466 patients was 58.3 years (range: 13.3–95.6 years): 359 (77.0%) were men; 367 (78.8%) had smear-positive pulmonary TB; 436 (93.6%) had never been treated for TB; 144 (30.9%) had cavitary lesions on chest radiograph; and 208 (44.6%) had bilateral lung parenchymal lesions.

Table 2 shows the proportion of patients with mixed infection in the different subgroups. The spline smoothing curve of the effect of age on risk of mixed infection after

TABLE 1. Correlation between the results of two amplification assays and mycobacterial culture for the detection of *Mycobacterium tuberculosis*

Assay	Specimen (no.)	No. of samples: Culture-positive for MTB (n = 486) Test +	Test −	Culture-negative for MTB[a] (n = 382) Test +	Test −	Sensitivity (%)	Specificity (%)
Cobas Amplicor	Sm+ sputum (563)	305	6	3	249	98.1	98.8
	Cul+ medium (305)	177	2	0	126	98.9	100
	Overall (868)	482	8	3	375	98.4	99.2
Q-PCR	Sm+ sputum (563)	292	19	5	247	93.9	98.0
	Cul+ medium (305)	174	5	1	125	97.2	99.2
	Overall (868)	466	24	6	372	95.1	98.4

Cul, culture; MTB, *Mycobacterium tuberculosis*; Q-PCR, quantitative PCR; Sm, smear.
[a]Culture-negative or non-tuberculosis mycobacterium.

TABLE 2. Proportion of patients with mixed infection in different subgroups

Characteristics		Total no. (%)	No. (%) of mixed infection	OR (95% CI)
Sample	Liquid culture medium	174 (37.3)	8 (4.6)	2.30 (0.78–6.74)
	Sputum	292 (62.7)	6 (2.1)	
Age (years)	>70	150 (32.2)	5 (3.3)	0.31 (0.07–1.38)
	25–70	286 (61.4)	6 (2.1)	0.19 (0.05–0.82)
	<25	30 (6.4)	3 (10.0)	
Sex	Women	107 (23.0)	6 (5.6)	2.60 (0.88–7.69)
	Men	359 (77.0)	8 (2.2)	
Smear grading	3+ to 4+	105 (22.5)	3 (2.9)	0.70 (0.15–3.20)
	1+ to 2+	262 (56.2)	7 (2.7)	0.65 (0.19–2.28)
	Negative	99 (21.2)	5 (4.0)	
Susceptibility	MDR	40 (8.6)	2 (5.1)	1.82 (0.39–8.54)
	Resistant but not MDR	45 (9.7)	1 (2.2)	0.77 (0.10–6.08)
	All susceptible	381 (81.7)	10 (2.6)	
Extent on CXR	Bilateral	208 (44.6)	8 (3.8)	1.68 (0.57–4.92)
	Unilateral	258 (55.4)	6 (2.3)	
Cavity(ies) on CXR	Yes	144 (30.9)	8 (5.6)	3.10 (1.05–9.10)
	No	322 (69.1)	6 (1.9)	
TB relapse	Yes	30 (6.4)	2 (6.7)	2.52 (0.54–11.83)
	No	436 (93.6)	12 (2.8)	

CXR, chest radiograph; MDR, multidrug-resistant; MGIT, TB, tuberculosis.

controlling for sex and pulmonary cavities showed a positive impact when age was <25 years or >70 years (see Fig. S2a). Therefore, patients were classified into three groups (Table 2).

Because of the presence of an interaction between age and pulmonary cavity, an interaction variable was included in the multivariate analysis for mixed infection (see Fig. S2b). In the multivariate analysis, only age <25 years with the presence of pulmonary cavities was associated with mixed infection (p 0.006; OR 9.6; 95% CI 1.9–47.5).

Factors influencing bacterial load of Beijing and non-Beijing strains

Among the 14 patients with mixed infection, the median age was 54 years, and there was a male/female ratio of 1.3 (see Table S1). The average bacterial loads of the Beijing and non-Beijing strains were 1100 and 84 copy, respectively. Beijing strains were the dominant strains in 13 (93%) patients. Among them, five (36%) had underlying comorbidity, and two (14%) had MDR TB. Within 1 year, 13 (93%) patients with mixed infection had been completely treated, as compared with 392 (87%) of the 452 patients without mixed infection (p 1.000 by Fisher's exact test).

In patients infected with Beijing strains (n = 245, including 231 infected with Beijing strains only and 14 with mixed infection), linear regression analysis revealed that the bacterial load of non-Beijing strains had no influence on that of the Beijing strain (Table 3). However in patients infected with non-Beijing strains (n = 235, including 221 infected with non-Beijing strains only and 14 with mixed infection), the bacterial load of Beijing strains was negatively correlated with that of non-Beijing strains. The presence of drug resistance was associated with the bacterial load of either Beijing or non-Beijing strain.

Discussion

The prevalence of mixed infection in TB patients is highly variable in different studies, ranging from 2.1% to 54% [3,5,12,19–26], with a trend showing that it parallels the local TB incidence. However, these results may not be directly comparable, owing to the limited number of patients (n = 9–249), the heterogeneity in study designs (different colonies from one isolate vs. serial isolates), and differences in methodology. In our study, the prevalence of mixed infection in culture-positive liquid medium samples from patients with pulmonary TB in Taiwan was 4.6%, much less than that (18.8%) in Cape Town [5], an area with a TB annual incidence (251 per 100 000 population) about four-fold higher than that in northern Taiwan (62 per 100 000 population) [27]. In Shanghai, China, an area with a TB annual incidence (38 per 100 000 population) less than that in Taiwan, the prevalence of mixed infection was 5.6% [23].

In previous studies, clinical details of the patients with mixed infection have usually been lacking. Only two have reported that mixed infection is more frequently observed among retreatment patients [5,23]. However, whether re-

TABLE 3. Linear regression analysis for factors influencing the bacterial load in patients infected with Beijing or non-Beijing strains

Dependent variable	Independent variable	Parameter estimate	Standard error	t-value	p-value	Variance inflation
Log (copy no. of Beijing strain) in patients infected with Beijing strain	Smear grading	0.32	0.05	−3.51	<0.001	1.017
	Resistant but not MDR	0.55	0.24	2.84	0.017	1.022
	Age >70 years with cavity	−0.65	0.29	2.15	0.025	1.005
Log (copy no. of non-Beijing strain) in patients infected with non-Beijing strain	Log (copy no. of Beijing strain)	−0.26	0.07	−3.51	<0.001	1.001
	Smear grading	0.16	0.06	2.84	0.005	1.000
	Resistant but not MDR	0.45	0.21	2.15	0.032	1.000

MDR, multidrug-resistant.
Log represents the logarithm of the number in parentheses to base 10.

treatment patients were relapse, defaulted or failure cases was not further defined. In our study, relapse of TB was not associated with mixed infection, probably because the risk of re-infection in Taipei is low or the prior course of anti-TB treatment had eradicated all TB bacilli before new infection occurred. The small number of patients in the study may also contribute to the lack of association between relapse and mixed infection.

The presence of cavities on the chest radiograph implies high bacillary load [28], and has been reported to be associated with a decreased interferon-γ response [29]. Both conditions imply advanced TB and a poor immune response [30]. The incidence of TB is usually low in populations <25 years of age. These young TB patients might have a genetic and immune predisposition for mixed infection. Another possibility is that young people have a higher risk of exposure to multiple sources of TB infection, especially in crowded and closed places such as internet cafés, pubs, or apartments.

The findings that Beijing strains were the very dominant subpopulation in 93% of our patients with mixed infection, and that the presence of a Beijing strain was significantly associated with a lower bacterial load of non-Beijing strains in patients infected with non-Beijing strains, suggest that Beijing strains may have a survival advantage [31]. However, further studies on fitness are necessary to confirm our findings and explore the genetic mechanisms.

Our analysis revealed that non- multidrug resistance was associated with a high bacterial load of either a Beijing or non-Beijing strain. Because acquisition of drug resistance often leads to a reduction in virulence [32], it is possible that a higher bacterial load will be needed for the patients to become symptomatic and to seek medical help.

Our study has several limitations. First, although sensitive, our Q-PCR method is likely to underestimate the prevalence of mixed infection. Variable-number tandem repeat typing with discriminatory loci would be an ideal approach. Discrimination between 'ancient' and 'modern' sublineages of Beijing strains by NTF locus analysis [33] or RD deletion analysis [6,34] is also important. Our Q-PCR method was unable to detect mixed infection by two Beijing strains or two non-Beijing strains. The use of only one isolate from each patient could further underestimate mixed infection. Second, the small number of patients with mixed infection in our study limits the statistical power to detect other risk factors for mixed infection, such as drug resistance and smear grading. Further large-scale studies are necessary. Third, for patients with mixed infection, drug susceptibility testing was not performed separately for their Beijing and non-Beijing strains. Furthermore, the serostatus of human immunodeficiency virus was not routinely checked in this study. However, this should not be a serious concern, as the incidence of human immunodeficiency virus infection was 7.7 per 100 000 population in Taiwan in 2008 and is currently decreasing [35].

Conclusion

We have developed a sensitive Q-PCR method to detect the presence of Beijing and non-Beijing strains simultaneously in smear-positive sputum samples and culture-positive liquid medium samples. Patients who are under 25 years old and have cavities on chest radiographs are prone to have mixed infection by a Beijing and a non-Beijing strain, and the results of drug susceptibility testing of their mycobacterial isolates should be interpreted carefully. Beijing strains seem to be more dominant than non-Beijing strains in patients with mixed infection.

Author Contributions

J.-Y. Wang drafted the manuscript, and together with C.-Y. Chiang, C.-J. Yu and L.-N. Lee, designed the study and interpreted the results. H.-L. Hsu and M.-C. Yu participated in data collection and analysis. F.-L. Yu and M.-C. Yu performed laboratory procedures. P.-C. Yang was the director responsible for general organization and instruction.

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Transparency Declaration

All authors declare no conflict of interest of any nature.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The primers and probes for detecting the Beijing and non-Beijing strains of *Mycobacterium tuberculosis* in the real-time quantitative polymerase chain reaction.

Fig. S2. The smoothing curve of the effect of age on the probability of mixed infection by using the spline smoothing model after controlling for sex and cavitation shows a positive impact when age was less than 25 or greater than 70(2A). The effect of pulmonary cavitations on the probability of mixed infection was different in the three age groups (2B).

Table S1. Clinical characteristics of the 14 patients with mixed infection.

Data S1. Supplementary Data.

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